Overexpression of SPARC obliterates the *in vivo* tumorigenicity of human hepatocellular carcinoma cells.

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Novelty and impact of this paper.

This work shows the ability of SPARC to exert novel antitumoral effects on human hepatocellular carcinoma cells. Cancer cells acquire a less aggressive-like phenotype

partially through the induction of a mesenchymal-to-epithelial transition. Transient forced SPARC overexpression in HCC cells led them more susceptible to standard chemotherapy. In addition, *in vitro* and *in vivo* results further suggest SPARC overexpression as an strategy for controlling HCC tumor growth.

Abbreviations used: HCC: hepatocellular carcinoma; SPARC: Secreted protein, acidic and rich in cysteine; TAM: tumor associated macrophages; MET: mesenchymal-to-epithelial transition; FBS, fetal bovine serum; TGF-β1, transforming growth factor beta 1; rSPARC, recombinant human SPARC.

ABSTRACT

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer-related death worldwide. Current treatments are extremely disappointing. SPARC (Secreted protein, acidic and rich in cysteine) is a matricellular glycoprotein with differential expression in several tumors, including HCC, which significance remains unclear. We infected HCC cells (HepG2, Hep3B and Huh7) with an adenovirus expressing SPARC (AdsSPARC) to examine the role of SPARC expression on HCC cells and its effect on tumor aggressiveness. The *in vitro* HCC cells substrate-dependent proliferation and cell cycle profile were unaffected; however, SPARC overexpression reduced HCC proliferation when cells were grown in spheroids. A mild induction of cellular apoptosis was observed upon SPARC overexpression. SPARC overexpression resulted in spheroid growth inhibition *in vitro* while no effects were found when recombinant SPARC was exogenously applied. Moreover, the clonogenic and migratory capabilities were largely decreased in SPARC-overexpressing HCC cells, altogether suggesting a less aggressive HCC cell phenotype. Consistently, AdsSPARC-transduced cells showed increased E-cadherin expression and a concomitant decrease in N-cadherin expression. Furthermore, SPARC overexpression was found to reduce HCC cell viability in response to 5-FU-based chemotherapy *in vitro*, partially through induction of apoptosis. *In vivo* experiments revealed that SPARC overexpression in HCC cells inhibited their tumorigenic capacity and increased animal survival through a mechanism that partially involves host macrophages. Our data suggest that SPARC overexpression in HCC cells results in a reduced tumorigenicity partially through the induction of mesenchymal-to-epithelial transition (MET). These evidences point to SPARC as a novel target for HCC treatment.

INTRODUCTION.

HCC is the sixth most common cancer and the third leading cause of cancer-related death in the world [1]. Unfortunately, the incidence and mortality associated with HCC is increasing steadily in USA as in Europe [2]. Current curative options can be applied to a paucity of patients and, in general, the prognosis of HCC is dismal due to underlying cirrhosis as well as to poor tumor response to chemotherapeutic regime [3, 4]. Therefore, novel therapies are urgently needed for advanced HCC [5].

There is a complex cross-talk between cancer cells and different tumor microenvironmental components, such as fibroblasts, endothelial cells, tumor associated macrophages (TAM) and matricellular proteins [6]. Accumulating evidence indicates that this dynamic cross-talk can modulate tumor cell capacity to invade and disseminate [6]. It is therefore highly relevant to assess whether and how those environmental factors are able to regulate tumor cellular processes in cancer disease. With this regard and especially in the case of HCC, in which cirrhosis is the underlying disease of most patients [4], the focus is placed on analyzing cellular events triggered by cell-matrix interactions.

SPARC, also named BM40 or osteonectin, is a secreted multifunctional matricellular glycoprotein involved in a wide number of biological processes during development, tissue repair and remodeling [7-10]. Among them, it was shown to inhibit cell cycle in fibroblasts and endothelial cells, by arresting cells at G1 [11]. In certain cell types, SPARC has also been shown to reduce cell adhesion to integrin-ligand coated surfaces due to disruption of focal adhesion complexes, causing inhibition of cell spreading [12]. SPARC is known to interact with several extracellular matrix components and to bind to, and modulate the expression and activity of several growth factors and matrix metalloproteinases [13-15]. For instance, SPARC has been shown to induce anti-

angiogenesis likely through blockade of the VEGF- and FGF-2 induced endothelial cell proliferation, or to block tumor stromal cell growth through inhibition of PDGF activity [16-18].

Changes in SPARC expression levels were evidenced in several malignant tumors of epithelial and non-epithelial origin [19-22]. Nevertheless, the role of SPARC in cancer is controversial and it seems to depend on the type of tumor [23]. For example, overexpression of SPARC in neuroblastoma [24], pancreatic adenocarcinoma cells[25], ovarian cancer [26], colorectal carcinoma [27] and acute myeloid leukemia [28] was associated with good prognosis. Induction of cancer cell apoptosis and enhanced sensitivity to chemotherapeutic drugs are mechanisms likely involved in the beneficial antitumoral effects observed upon SPARC enhanced expression in colon cancer cells [29].

In spite of beneficial effects of SPARC overexpression in certain tumor types, the induction of SPARC has been positively associated with increased aggressiveness in melanoma [30, 31], glioblastoma [32], prostate [33] and breast cancer [34] . It seems that SPARC effects on different cancer types might depend on whether SPARC acts directly on tumor cells or indirectly, by influencing adjacent stromal cells [23].

The expression of SPARC was shown to be induced in myofibroblasts of cirrhotic livers in HCV chronically infected patients [35] and in experimental liver cirrhosis [36]. Similarly, SPARC overexpression was observed in HCC, along capillaries present in the tumor capsule [37]. Lau et al. showed HCC antitumoral effects of SPARC forced expression in human tumor xenografts which correlated with a decreased neo-angiogenesis [22], but no mechanism was proposed. Thus, even though some authors showed SPARC overexpression in HCC [22, 35, 37, 38] and others reported some antitumor effects[22] the significance of SPARC expression changes in HCC still remains unclear and the mechanisms involved, unknown.

Cadherins are a superfamily of transmembrane glycoproteins that mediate intercellular adhesion as well as other signaling events [39]. Disruptions in E-cadherin expression have been associated with enhanced aggressiveness of certain types of tumors including HCC [40-42]. Moreover, enhanced expression of mesenchymal-associated cadherins, such as N-cadherin, in cancer cells increased tumor cell aggressiveness and facilitated tumor dissemination [43].

In an effort to assess the role and action mechanisms of SPARC in HCC, we transiently overexpressed SPARC in HepG2 HCC cells by transduction with an adenoviral vector (AdsSPARC). Our results hereby show for the first time that a transient increase in SPARC expression levels on HCC cells reduced their spheroid growth, clonogenic, migratory and adherent capabilities. The *in vivo* growth capacity of SPARC-overexpressing HCC cells was strongly inhibited in nude mice, a feature now associated with an increased number of host macrophage cells. Interestingly, up-regulation of SPARC expression decreases HCC cell viability in response to 5-FU-based chemotherapy. More importantly, overexpression of SPARC was associated with an up-regulation of E-cadherin and a consistent down-regulation of N-cadherin, a tandem that likely results in a less aggressive HCC cell phenotype.

MATERIAL AND METHODS.

Generation of recombinant adenoviral vectors.

AdsSPARC, a first-generation replication-defective adenovirus was constructed and produced as previously described [36]. Briefly, a 1.7 kb *Sal*I fragment containing the coding sequence of human SPARC or a 527 bp *Sal*I fragment containing the bacterial β-galactosidase gene were cloned in pADPSY-LTRSVpolyA vector to generate adenoviral vectors carrying SPARC cDNA in sense orientation (AdsSPARC) or Ad-βgal, respectively. AdsSPARC and Ad-βgal were expanded in HEK-293 cells, purified by cesium chloride density gradient, desalted using a PD-10 Sephadex G-25 column (Amersham Biosciences, Piscataway, NJ), and stored at -80°C. The concentration of recombinant vector was expressed as 50% tissue culture infectious doses (TCID50) per milliliter [44]. For SPARC downregulation experiments, adenoviral vectors carrying SPARC cDNA in antisense orientation (AdasSPARC) were generated and experimentally applied [36].

Cells and cell culture.

HepG2, Hep3B and HuH7 human hepatocellular carcinoma cells (kindly provided by Prof. Prieto, University of Navarra) were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS) and antibiotics. Cell cultures were maintained at 37C in a 5% CO_2 humidified incubator. HCC cells, transduced with AdsSPARC, AdasSPARC or Adβgal at MOI of 100 or non-transduced cells were used in different experiments.

Immunofluorescence assays.

For immunofluorescence studies of SPARC expression HepG2 cells were fixed in 4% paraformaldehyde and blocked with 10 % goat serum in phosphate buffered saline (PBS)-0.2%Tween for 60 min at room temperature, followed by overnight incubation at 4ºC in a humid chamber with a polyclonal rabbit anti-SPARC antibody (32 µg/mL; Hybridoma). After 3 washes with PBS, bound antibodies were detected with FITC-conjugated goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA). Secondary antibody was diluted 1:40 in PBS-0.2% Tween and incubated for 2h at 37ºC. Nuclear morphology was examined by staining with DAPI. Images were captured from a Nikon E800 microscope coupled to a CCD camera. Control experiments without primary antibody showed only a faint background staining (not shown).

Three-dimensional spheroids

Ninety-six-well tissue culture plates were coated with 75 µl of 1% agarose in PBS. Nearly confluent non-transduced HCC, HCC/AdsSPARC, HCC/AdasSPARC or HCC/Ad-βgal (HepG2 and Hep3B) were washed twice with PBS, trypsinized, and seeded at $5x10^3$ cells/well in 150 µl of 2% FBS DMEM to obtain a single homotypic spheroid per well. Seventy-five microliters of supernatant were carefully removed from each well every 3 days and replaced with fresh medium. Spheroid size was measured at days 2 and 6 using an inverted microscope and photographed. Length and width were measured using the ImageJ program (NIH). Spheroid volume was expressed as arbitrary units. Non-infected HepG2 cells were seeded at $5x10^3$ cells/well in 150 µl of 2 % FBS DMEM with or without 0,5 µg/ml of recombinant SPARC. Spheroids volume was measured as described above

Proliferation assays and in vitro apoptosis assessment.

Cell proliferation was measured using the colorimetric MTT assay (Invitrogen). Briefly, $3x10^3$ HCC cells (HepG2, Hep3B and Huh7)/well were seeded onto 96-well plates in a final volume of 100 µl per well. At each time point, culture medium was replaced with 100 µl of 5 mg/ml 3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyl tetrazolium bromide. Four hours later, the formazan dye was solubilized and read at 490nm optical density as described (Denizot and Lang, 1986). Each assay was performed three times in triplicate. HepG2 spheroids were trypsinized and proliferation was similarly analyzed. For in vitro chemotherapy assays, HepG2 cells were transduced with AdsSPARC, Ad-βgal or left non transduced for 2 days, washed and further incubated for 24 h with no 5-FU or with different doses of 5-FU ranging from 0.1 to 10 µg/ml. Cell viability was assessed by MTT assay as described above.

Morphological changes associated with apoptosis were assessed by acridine orange-ethidium bromide mixture staining (Sigma). Single cell suspensions were stained with 10 µg/ml of the mixture and cells were visualized under a fluorescence microscope. Apoptotic cells were defined as those stained in yellow and showing cytoplasmic and nuclear shrinkage and chromatin condensation or fragmentation. At least 100 cells were counted from 4 independent experiments and the percentage of apoptotic and necrotic cells was determined. Apoptosis analysis was performed on HepG2 cells infected with AdsSPARC, Ad-βgal or left uninfected by flow cytometry at 48 h using an apoptosis detection kit based on annexin-V staining (eBioscienceTM, SanDiego, CA). Briefly, HepG2 cells ($1x10^6$) were collected with EDTA, washed twice in PBS, centrifuged and incubated with 5µl of annexin-V in binding buffer at room temperature for 15 min. Cells were vortexed and centrifuged, and the resultant pellets were washed and stained with 1µl propidium iodide in binding buffer. The percentage of apoptotic cells were immediately analyzed in a FACSCalibur (Becton Dickinson). Results are representative

of 4 different experiments. For quantifications, mean values from each independent experiment were normalized to their respective control values, prior to statistical comparisons.

Cell cycle analysis

For cell cycle analysis, $2x10^6$ cells were collected, washed in PBS and fixed in a mixture of ice-cold 70% (v/v) ethanol, FBS and distill water. Fixed cells were centrifuged and stained with propidium iodide (PI) solution (50 mg/ml PI, 180 U/ml RNAse). DNA content was determined using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Colony formation and cell migration assay.

For colony formation assay, $1x10^3$ HCC cells (HepG2, Hep3B and Huh7) left untreated (control) or transduced with AdsSPARC, AdasSPARC or Ad-βgal were plated onto 60-mm dishes and incubated for 2 weeks before staining with crystal violet. Colonies, composed by 20-25 cells, was quantified under phase-contrast light microscopy. Three independent experiments were performed, in triplicates. For transwell cell migration assays, non-transduced or AdsSPARC or Ad-βgal transduced HepG2 or Huh7 cells ($5x10^4$ in 100 µl of 0.1% FBS DMEM) were seeded on the upper chambers of 48-well chamber plates (Neuroprobe). In the lower chamber, 5 ng/ml TGF-β1 was added as the chemoattractant. After 16 hours incubation at 37°C in 5% CO_2, the cells that remained on the upper surface of the membrane were removed by wiping with a cotton bud. Migrated cells attached to the lower surface of the membrane were fixed with 2% formaldehyde/PBS and stained with 10% May Gründwald-Giemsa. The number of migrated cells on each membrane was counted under a microscope (x100), for ten

random microscopic fields per membrane, and averaged. In order to analyze the differential adhesive capacity of treated cells onto polycarbonate membranes, HepG2 cells were seeded on the upper chambers and four hours later, the cells attached to the upper surface of the membrane were fixed and stained.

Western blot analysis.

Human SPARC was detected in supernatants by monoclonal anti-SPARC antibody (diluted 1:500). Supernatants were collected and centrifuged twice at 4ºC for 20 min at 10000 x g. After centrifugation, cleared supernatants were stored at -80ºC until analysis. E-cadherin was detected in HepG2 extracts by mouse monoclonal anti-E-cadherin antibody (diluted 1:2000; kindly provided by Dr Berasain,University of Navarra). Briefly, cells were collected and incubated in lysis buffer with protease inhibitors (50mM Tris-HCI buffer, ph7.4, containing 0.1%Tween-20, 150mM NaCl, 10µg/ml aprotinin, 5µg/ml leupeptin, 1mM PMSF) 30 min on ice. Measurement of total protein concentration was performed using Bradford assay [45]. For immunoblotting, 100 µg of total protein was loaded and separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes as described previously [31]. Blots were then developed with HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:5000 in blocking buffer [31]. Bands were detected using the ECL detection system. Protein loading and transfer for E-cadherin was monitored using an anti-actin antibody (diluted 1/700, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (diluted 1/5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). For SPARC protein loading membranes were stained with Ponceau Red. Bands intensities were measured by densitometer analysis using the Scion Image software (Scion Corporation, USA).

N-cadherin flow cytometry.

For flow cytometry analysis, HepG2 cells grown for 48 and 72 h in medium supplemented with 2% FBS were washed and detached with 1.25 mM EDTA in PBS. After washing with ice-cold medium, cells were incubated with 2 mg/mL of a monoclonal anti-N-cadherin antibody (anti-A-CAM, clone CG-4, Sigma, St. Louis, MO) for 30 min, washed twice with PBS 0.1% BSA. After washings, samples were incubated for 30 min with a secondary goat FITC-conjugated anti-mouse IgG antibody (Jackson Immuno Research, West Grove, PA) and fixed in 4% w/v paraformaldehyde in PBS for 10 min at room temperature. Cells were washed, resuspended in PBS and subjected to flow cytometry using a FACSCalibur (Becton Dickinson) flow cytometer.

Animal studies.

For assessment of the *in vivo* tumor growth, HepG2 or HuH7 HCCs were left untransduced or were *ex vivo*-transduced with AdsSPARC, AdasSPARC or Ad-βgal, at a MOI of 100. Twenty hours later, cells were trypsinized, counted, and resuspended in 100 µl of saline. Six- to 8-week-old male athymic N:NIH(S)-nu mice were subcutaneously injected into the right flank with $1.5x10^6$ HepG2 cells or $5x10^6$ Huh7 cells. Perpendicular diameters were used to determine tumor volume (V=(dlxds2x0.52), where ds is the smaller diameter and dl is the larger one. Ten animals were allocated to each treatment group. For histological analysis, tumor tissue was obtained at day 5 post HCC cells inoculation. All procedures were performed according to the "Guide for the Care and Use of Laboratory Animals" published by the U.S. National Research Council (National Academy Press, Washington, D.C. 1996) and approved by the School of Biomedical Sciences of the Austral University.

Histology and immunocyto/histochemistry

Tumor sections from individual mice were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin for morphological evaluation. Five-micrometer-thick tumor sections were de-paraffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by incubating with 3% hydrogen peroxide in methanol for 20 minutes. Nonspecific primary antibody binding was blocked by incubating sections in normal goat serum (10% in PBS). Endogenous biotin and avidin was blocked with blocking agent complex (Vectastain ABC Elite; Vector Labs., Burlingame, CA). For detection of macrophages, tissue sections were incubated with a rat anti-F4/80 monoclonal antibody (1:100; Serotec) overnight at 4ºC in PBS containing 0.1% triton 0.1% bovine serum albumin. Slides were incubated with peroxidase-linked biotinylated goat anti-mouse secondary antibodies for 60 min, washed and further incubated with the ABC kit (Vector Laboratories). Sections were washed and incubated in a mixture of 3.3-diaminobenzidine (DAB) and *nickel-ammonium salt* for enhancement of the signal [46]. Controls for immunostaining specificity in which the primary antibody was replaced by non-immune mouse serum or omitted, were negative. Quantitative analysis of immunohistochemical staining for F4/80 was performed using the ImageJ software, from 400x magnification images.

For N-cadherin and smooth muscle actin (SMA) immunocytochemistries, 1000 HepG2 cells/well were plated onto poli-L-lysine/fibronectin coated coverslips placed on 24-well plates for 8 hours and then fixed in 4% paraformaldehyde. Cells were incubated overnight with a mouse anti-N-cadherin antibody (1:50; Zymed) or a mouse anti-α-SMA (1:200, Sigma), and specific antibody binding was evidenced by incubation with a donkey anti-mouse secondary antibody (1:50; Jackson Immunoresearch) followed by

chromogenic reaction (ABC kit, Vector Laboratories) with nickel salt enhancement of the signal [46]. At least two independent experiments were performed, with similar results. For quantifications, only spread cells were considered. The N-cadherin and α-SMA immunostaining intensities were measured using the ImageJ software by analyzing 20 randomly sampled cells per condition. For this purpose, a mean intensity value per cell was obtained from 5 random values taken from similar areas of the cytoplasm. Cell morphology was determined by establishing similar cultures of HepG2 cells after 16 hours of incubation.

In vivo apoptosis. TUNEL assay.

For apoptosis assay, 5-micrometer-thick tumor sections were fixed with 10% formalin for 20m and the terminal deoxynucleotidyl transferase–mediated DUPT nick-end labeling (TUNEL) assay was done following the manufacturer's protocol (Fluorescein-FragEL™ DNA Fragmentation Detection Kit, Calbiochem, Darmstadt, Germany). TUNEL-positive cells were analyzed by using a standard fluorescein filter (465-495) and were viewed with a microscope (Nikon).

Statistical analysis.

Data are expressed as mean ± SEM. Statistical analysis was performed using Student's *t,* Mann-Whitney tests or Kruskal-Wallis, when appropriate. Differences at $P<0.05$ were considered to be significant. Survival rates were calculated with the Kaplan–Meier method and their differences were evaluated by the log-rank test. Data analysis was performed with the Prism GraphPad (GraphPad Software, Inc., San Diego, CA).

RESULTS.

Endogenous SPARC overexpression inhibits HCC cell spheroid growth.

As shown by western blot (Fig. 1A) and by immunofluorescense (Fig. 1B), SPARC expression was identified in HepG2 cells. AdsSPARC transduction (MOI 100) resulted in a significant SPARC overexpression at 3 days after gene transfer (Figure 1A).

To establish the role of SPARC overexpression in HCC cells we initially performed *in vitro* studies growing HCC cells in tri-dimensional spheroids. A profound inhibitory effect on spheroid growth of HepG2 cells was observed following SPARC overexpression (Figure 2A, upper panel; 2B). On the contrary, transduction of HCC cells with Ad-βgal or AdasSPARC had no effects on spheroid growth capacity comparable to untransduced cells. Interestingly, when HepG2 cells were exogenously treated with recombinant SPARC (Figure 2C) spheroid growth rate was not affected, indicating that only endogenous increased levels of this protein was able to induce the inhibitory effects on HCC growth. A similar inhibition in spheroid growth formation was found in SPARC-overexpressing Hep3B cells (Figure 2A, lower panel).

Increased endogenous SPARC expression levels do not affect proliferation of HCC cells.

To examine whether SPARC overexpression on HCC cells (HepG2, Hep3B and Huh7) could influence tumor cell substrate-dependent proliferation capacity, cells were assessed for their *in vitro* growth capacity in plastic. No significant differences were found in between groups up to 72 h (Figure 3A). Similar results were observed at 120 h (not shown). We next aimed to address whether AdsSPARC transduction on HCC cells could induce apoptosis. For this purpose, HepG2 cells were stained with acridine orange-ethidium bromide mixture solution and they were subsequently visualized under

immunofluorescence microscope. SPARC overexpression resulted in a mild and non-significant increased induction of cellular apoptosis (Fig. 3B). However, this effect was shown to be significant by flow cytometry when the annexin V staining was used instead (Fig. 3C). Thus, in order to confirm the lack of significant effects of SPARC overexpression on HCC cell proliferation and to address the extent of apoptosis events, the pattern of cell cycle progression was analyzed. No differences were found among treatments (Fig. 3D). Similarly, when cell cycle analysis was performed on Hep3B cells no differences were found in between conditions (G1 phase= 55.5%, 61% and 66%; S phase = 35.6%, 31% and 24.5%; G2 phase= 8.9%, 7.2% and 9.1%; for untransduced, Ad-βgal and AdsSPARC transduced cells, respectively). Since both the proliferation rate and the cell cycle pattern remained unaffected, we conclude that SPARC overexpression-mediated increase in HCC cellular apoptosis was not remarkable in terms of substrate-dependent cell growth.

We next wonder whether substrate-independent growth of HCC cells might be influenced by SPARC overexpression. To address this issue, HepG2 cells were grown in spheroids. Interestingly, and contrarily to what we have observed in monolayer-cultured HCC cells, a significant reduction in proliferation activity was observed in spheroids composed by AdsSPARC-treated cells (Fig. 3E). In order to exclude the partial involvement of indirect mechanisms mediated by cellular apoptosis, HepG2 spheroids were dissociated and stained with acridine orange-ethidium bromide mixture solution. No significant differences were found, which overall suggests that an increase in endogenous levels of SPARC in HCC cells reduces their substrate-independent proliferation capacity, therefore resulting in a partial inhibition of their tridimensional growth capacity.

Clonogenic and migratory capabilities of HCC cells are inhibited by SPARC over-expression. Possible implications on HCC cell aggressiveness.

To assess whether SPARC overexpression in HCC cells might affect their colony formation capacity, a feature of cell aggressiveness, cells were plated at a density of 10^3 cells per 60-mm dish. After 15 days of incubation, the number of colonies was significantly decreased (16-fold) in cells with forced SPARC overexpression when compared with Ad-βgal- or AdasSPARC-treated cells (Figure 4A).

Considering the ability of SPARC to modulate adhesiveness of certain tumor cells, we next wonder whether the increased SPARC expression in HCC cells could affect cell adhesiveness. For this purpose, cells were placed on fibronectin for 4 hours. AdsSPARC overexpressing cells showed a reduced ability to adhere to fibronectin (data not shown).

Migration is considered to be a critical mechanism in tumor cell dissemination. SPARC was previously shown to act as a chemotactic factor for prostate and breast carcinoma cell migration towards bone extracts [47, 48]; nevertheless, endogenous SPARC expression was reported to have opposite effects in glioma cells [49] or to render no effects on MDA-MB-231 breast cancer cells [50]. Therefore, we decided to explore whether overexpression of SPARC might affect HCC cells chemotactic capacity towards TGF-β1. As shown in figure 4B, AdsSPARC-HepG2 cells exhibited significantly less chemotactic migration capacity than cells transduced with Ad-βgal or untransduced cells. Similar results were obtained when Hep3B cells were used instead (not shown). Importantly, no differences in transwell membrane adherence of HepG2 cells were found among conditions. Therefore, the reduced migration capacity of SPARC-overexpressing cells seems not to be principally related to differential adherence of HCC cells (Fig.4B upper).Thus, overall these data, including colony formation capacity, cell adhesiveness and cell

migration capacity results, suggest that intracellular SPARC overexpression likely diminishes the aggressive-like behavior of HCC cells.

E-cadherins are involved in maintaining the epithelial structure of a number of tumor types and their down-regulation was associated with an increased invasiveness of human hepatocellular carcinomas [51, 52]. In addition, N-cadherin expression is frequently induced in highly aggressive tumors [43]. To further investigate the potential involvement of cadherins in SPARC-induced effects, changes in both E- and N-cadherin expression levels were investigated. Western blot analysis of AdsSPARC-HCC cells showed increased E-cadherin expression levels at day 3 (Fig. 5A). On the other hand, a 50% reduction in N-cadherin cell surface expression levels was observed in AdsSPARC-transduced HepG2 cells when compared to Ad-βgal-transduced or untransduced cells (Fig. 5B and C). A similar degree of N-cadherin inhibition was found in SPARC-overexpressing HCC cultured cells by immunocytochemistry (Figure 5D-H). To confirm the previous result, we have performed another experiment and immunostained cultures against α-SMA, another mesenchymal marker. Consistently, α-SMA expression was also inhibited in AdsSPARC-treated cells (Figure 5I). From previous results, we conclude that SPARC overexpression in HepG2 cells results in the upregulation of E-cadherin levels and in the downregulation of N-cadherin as well as of α-SMA levels, which further suggest they have acquired a less aggressive phenotype. In addition, the finding of cell rounding in HCC overexpressing SPARC could be the result of its counteradhesive properties.

Upregulation of SPARC expression decreases HCC cells viability and increases their apoptosis in response to 5-FU-based chemotherapy.

HCC cells are naturally resistant to any chemotherapeutic drug. One of the proposed mechanisms responsible for the observed antitumoral effects of SPARC is its ability to induce apoptosis of cancer cells [29]. In order to assess whether SPARC overexpression might led HCC cells more susceptible to chemotherapeutic agents, we tested the effects of 5-FU and adriamycin on HepG2 cells proliferation capacity and cell apoptosis. SPARC overexpression resulted in an increased sensitivity to 5-FU chemotherapy since both a 50% decreased cellular viability (Fig. 6A) and an increased apoptosis (~70% at 10 µg/ml 5-FU) (Fig. 6B) were observed. No changes were found when adriamycin was used as a chemotherapeutic agent instead (not shown).

The *in vivo* growth capacity of SPARC-overexpressing HCC cells is strongly inhibited in nude mice.

We aimed to explore whether transient SPARC overexpression in two different HCC cell lines might affect their *in vivo* growth. Nude mice injected with control HepG2 cells showed an average tumor volume of 500-600 to 900 mm^3 at day 40. Contrarily, transient overexpression of SPARC in HepG2 cells significantly inhibited tumor growth (reaching an average maximum tumor volume value of 30 mm^3) (Figure 7A) and significantly increased animal survival (log rank test $p<0.05$) (Figure 7B). No effects *in vivo* were observed on tumor growth upon transient SPARC downregulation. Similar results although less potent resulted when Huh7 HCCs were used instead, with an increased in animal survival (Figure 7C and D). H&E staining of HepG2 tumors overexpressing SPARC showed a decreased rim of viable tumor cells in comparison with tumors of untransduced or Ad-βgal- or AdasSPARC treated cells (Figure 8A and not shown). In addition, we have observed a reduced number of cells with fibroblast morphology in the AdsSPARC-HCC treated group in comparison with controls.

Over-expression of SPARC in HCC cells results in increased macrophage infiltration.

Considering the importance of macrophages in the progression of tumors, we assessed their possible involvement in the *in vivo* observed SPARC effects. Interestingly, the number of infiltrating macrophages ($F4/80^+$ cells) was significantly higher in AdsSPARC-HepG2 HCC tumors in comparison with control animal groups (F4/80 labeled area: 56463±7209 vs. 17072±2767 vs. 20785±2634 $pixels^2$; AdsSPARC vs. Ad-βgal vs. Control; $p<0.0001$; Fig.8B). Significantly higher numbers of F4/80-positive cells were found in the core region of tumors from AdsSPARC-treated animals than in those from mice injected with HepG2 or Ad-βgal HepG2 cells (Figure 8D). This feature could be a consequence of enhanced tumor cell apoptosis, since an increase in apoptosis induction was observed by TUNEL in SPARC overexpressing tumors (Figure 8C).

DISCUSSION.

In this paper, we provide for the first time, clear evidence that SPARC has a role on the invasive potential of HCC. Transient, overexpression of SPARC in HCC cells transduction resulted in a phenotype indicative of decreased tumor cell aggressiveness. Moreover, increases endogenous SPARC expression in HepG2 cells resulted in: i) reduced capacity to form three-dimensional spheroids and decreased substrate-independent proliferation, ii) decreased ability to migrate and to generate cellular colonies; iii) increased expression of E-cadherin and a concomitant decrease in N-cadherin expression, iv), increased susceptibility to 5-FU-based chemotherapy and v) a potent inhibition of tumorigenicity *in vivo* leading to increased animal survival. The results from key experiments were also confirmed in other two HCC cell lines (Hep3B and Huh7) demonstrating that these findings do not correspond to cell specific phenomena.

HCC is a complex disease that often progresses as a consequence of genetic anomalies in cancer cells affecting many cell-growth regulatory pathways and also as a result of tumor cell interactions with microenvironmental factors [53]. In this context, we have addressed the role of SPARC, a matricellular glycoprotein with a complex regulatory function associated with increased aggressiveness in a number of human cancers [54]. There is contrasting evidence in the literature regarding SPARC influence on cancer cell behavior [54]. Although SPARC has been shown to be expressed by cancer cells, in certain tumors it is produced as well and at much higher levels by fibroblasts and endothelial cells [55]. SPARC is almost undetectable in normal hepatic tissue by immunohistochemistry, whereas substantial induction of its expression is observed in the HCC stroma [37]. Our data indicate that only endogenous increased levels of SPARC could exert a beneficial effect on HCC growth. Even though there is abundant

information on the role of SPARC in other cancer types [23], little is known about its involvement in hepatocarcinogenesis.

In order to analyze the effects of increasing SPARC expression levels on HCC cells we chose to take advantage of available gene therapy vectors [56]. Among them, replication deficient-recombinant adenoviruses have been widely used for gene transfer to the liver and especially for HCC experimental treatment [57-59]. In particular, Type 5 adenoviruses very efficiently infect hepatic cells including HCC cells [59, 60].

SPARC has been previously shown to induce the inhibition of cancer cells proliferation[61], arresting cells at G_0. The mechanisms by which SPARC inhibits proliferation were previously associated with alteration of growth factor signaling events through diverse mechanisms including interaction with PDGF receptors [62] with the result of Mitogen-Activated Protein Kinase (MAPK) inhibition, cyclin E-Cyclin Dependent Kinase 2 (CDK2) inactivation, cyclin A down-regulation, and maintenance of RB activation [18]. Anti-proliferative effects of SPARC were also associated with indirect effects on IGF signals [63]. Interestingly, we show here that SPARC overexpression in HCC cells did not affect substrate-dependent cell proliferation. In addition, SPARC overexpression has been shown to slightly induce cellular apoptosis in HepG2 cells. It is noteworthy that the AdsSPARC-mediated induction of SPARC inhibited spheroid formation of HCC cells, whereas its knock-down using SPARC antisense mRNA had no effects on spheroid growth. Moreover, when cultured as monolayer and even though cellular growth was unaffected by SPARC overexpression (not shown), a clear inhibitory effect on their clonogenic capacity was noted. Furthermore, no effects on spheroid growth were observed when recombinant SPARC was exogenously applied, indicating that SPARC effects on HCC tumor cells largely rely on the modulation of its cellular levels.

Migration is a key step for the invasive and disseminating capabilities of cancer cells. In our study, forced upregulation of SPARC expression inhibited migration of HCC cells towards TGF-β1, used as a chemoatractant. Although the mechanisms have been unknown, some evidences in glioma cell lines suggested that cell migration capability may depend on the type of extracellular matrix proteins present in their microenvironment (41).

One of the key processes providing cancer cells with the capacity to migrate, invade and metastasize is their ability to undergo an epithelial to mesenchymal transition (EMT) [64]. EMT is characterized by loss of intercellular adhesion (E-cadherin to N-cadherin swicht) [64], downregulation of epithelial markers (cytokeratins), upregulation of MMP-2 and MMP-9 and the acquisition of a fibroblast-like motile phenotype. In fact, the induction of an EMT has been implicated in the malignant progression of HCC [41, 42]. We observed that SPARC overexpression resulted in E-cadherin upregulation and N-cadherin downregulation as well as in a reduced expression of α-SMA protein altogether suggesting the involvement of mesenchymal to epithelial transition (MET) events. However, no significant changes were observed on MMP-2 and MMP-9 activities which might be due to low frequency of EMT events in HepG2 cells as suggested by the low levels of N-cadherin expression found in this specific cell line (not shown). These data are in contrast with our and others previous observations in melanoma cells, in which high levels of SPARC are associated with N-cadherin upregulation and E-cadherin downregulation [65, 66]; however, they are consistent with the increase of tumor aggressiveness observed in other melanoma models when SPARC is overexpressed [30, 31] Thus, SPARC overexpression in different kinds of cancer cells seems to be differently involved in modulating EMT or MET, likely affecting cells migratory properties. Together, our data suggest that SPARC overexpression in HCC

cells induces a MET in HCC cells, a mechanism which is likely involved in differences in cell behavior hereby shown, which are indicative of a less aggressive-like phenotype.

Many chemotherapeutic agents have been applied in the treatment of HCC [67]. High doses of chemotherapeutic drugs are necessary to obtain a low rate of response. One of the chemotherapeutic agents most extensively studied is 5-FU, a pyrimidine antimetabolite which has an overall response rate lower than 10% [67]. Chemotherapy in cirrhotic patients is poorly tolerated and it is no longer applied to patients with advanced or metastatic HCC [67]. It is clear that there is a need for new non-toxic agents or new strategies that can re-sensitize HCC cells to standard chemotherapy. Based on the present data SPARC appears to function as a tumor suppressor in some cancer cells and one of the mechanisms is the induction of cellular apoptosis. For instance, SPARC has been shown to reverse chemotherapy resistance in colorectal carcinoma cells through induction of caspase-8 activation [29]. Consistent with those results, we found that SPARC overexpression in HCC cells resulted in a decreased proliferation and increased apoptosis when cells were subjected to lower concentrations of 5-FU. This evidence provides a proof-of-principle that the reversal of resistance to 5-FU-based chemotherapy can potentially be exploited therapeutically for HCC. Thus, increasing SPARC expression on HCC cells through gene therapy could be an attractive strategy to render cells more susceptible to standard chemotherapy or to combined treatments.

Confirming previous results [22], nude animals inoculated with SPARC-overexpressing HCC cells showed a strong inhibition of tumor and significant increase in long-term survival when compared to controls. In agreement with *in vitro* effects, knock-down of SPARC had no effects on *in vivo* tumor growth. It still remains unclear to a certain extent why SPARC is able to inhibit tumor growth in certain types of tumor while in others it has the opposite effect [23]. The timing of SPARC expression might partially

explain those contrasting results; thus, in our work, high but transient SPARC overexpression seems to be a sufficient stimulus to decrease the aggressive-like behavior of HCC cells. The induction in E-cadherin and downregulation of N-cadherin by SPARC overexpression might be partially involved in the decreased growth ability of tumor cells likely through interfering with other microenvironmental factors in the cell-cell context. We observed that the density of host tumor invading macrophages is increased in AdsSPARC-HCC cells treated animals. This feature might be the consequence of *in vivo* apoptosis induction in SPARC overexpressing HCC cells and/or it could be an active event mediated by the enhancement in SPARC expression. Another particular observation emerging from histological analysis of HCC tumors is the reduced number of cells with fibroblast morphology in the AdsSPARC-HCC treated group, suggesting that SPARC might have a role in extracellular matrix organization and/or fibroblast invasiveness to tumor mass periphery. On the other hand, only mild changes in tumor vasculature were observed after CD31 or von-Willebrand immunostainings, at 5 days after tumor cell injection, although it is well known that SPARC has antiangiogenic properties.

In summary, our study implicates SPARC as a HCC tumor modulator protein able to inhibit volumetric tumor growth both *in vitro* and *in vivo* and to influence several other morphological and behavioral cellular features intimately linked to cancer cell aggressiveness. In addition, we show that SPARC overexpression in HCC cells likely increase tumor cells sensitivity to 5-FU-based chemotherapy. For previous reasons, the enhancement of SPARC expression in HCC cells is proposed as a potential strategy for the treatment of HCC, a disease in which other therapeutic approaches are nowadays mostly neglected.

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FIGURE LEGENDS.

Figure 1. Overexpression of SPARC by AdsSPARC.

Western blot analysis of SPARC on HepG2 hepatocellular carcinoma cells. AdsSPARC (at MOI 100) induces high levels of secreted SPARC. **A)** Western blot of conditioned media obtained from HepG2 cells following transduction with the different adenoviral vectors. Samples were collected at day 3 after cell transduction. **B)** Immunofluorescent staining of HepG2 naïve cells. Fluorescence signals specific to SPARC antibody are visualized in green and nuclei, in blue (DAPI staining).

Figure 2. Positive modulation of SPARC levels strongly inhibits HCC multicellular spheroid growth.

A) Spheroids prepared from HepG2 (upper panel) and Hep3B (lower panel) cells were transduced with AdsSPARC, AdasSPARC or Ad-βgal. Comparisons of spheroid mean volumes at days 2 and 6. Data expressed as the mean ± SEM correspond to 3 experiments performed in 5 replicas. (*) vs. Ad-βgal. (*) <0.05 using Mann-Whitney test. **B)** Photographs of the spheroids from HepG2 cells at day 6, taken under phase-contrast light microscopy. **C)** Exogenous administration of SPARC as a recombinant protein did not affect spheroid growth on HepG2 cells. Kruskal-Wallis showed no significant differences among groups.

Figure 3. SPARC upregulation does not profoundly affect HCC cell growth in vitro.

A) HCC cells (HepG2, Hep3B and Huh7) were incubated with no addition of viral vectors or with AdsSPARC, AdasSPARC or Ad-βgal for 3 days, at a MOI of 100. Cell viability was determined by MTT assay (Invitrogen) at 490 nm, in 3 independent

studies; ns: non-significant. (*) $p<0.05$ using Mann-Whitney test. **B)** SPARC overexpression results in a non-significant increase in HepG2 cellular apoptosis when analyzed by the acridine-orange/ethidium bromide method. (*) $p<0.05$ using Mann-Whitney test. **C)** Flow cytometric analysis of HepG2 cells stained with annexin-V obtained after 72 hours in culture showed a slight significant induction in cellular apoptosis in SPARC overexpressing cells. Results are representative of 4 experiments similarly performed. (*) $p<0.05$ using Mann-Whitney test. **D)** Cell cycle analysis from DNA content. Cell cycle profiles correspond to one representative experiment of the three that were performed. Values are expressed as mean ± SEM. **E)** Proliferation activity of cells grown in spheroids, analyzed by MTT. (*) $p<0.05$ using Student's *t* test.

Figure 4. SPARC profoundly influences colony formation and migratory capability in HCC cells.

A) AdsSPARC reduced HCC cell clonogenic capacity. For colony formation assay 10^3 untreated (Control) or AdsSPARC, AdasSPARC or Ad-βgal transduced (MOI 100) HCC cells (HepG2, Hep3B and Huh7) were cultured in a 6-well plate for 2 weeks before crystal violet staining, and the number of colonies (20-25 cells) was quantified under phase-contrast light microscopy (*) $P < 0.05$ vs. Control; Mann-Whitney test. **B)** AdsSPARC inhibits migration towards TGF-β1 of HepG2 cells. HepG2 cells were transduced with AdsSPARC or Ad-βgal or left untransduced for 3 days at a MOI of 100. Migration assays were performed in the absence of FBS as described in M&M. Transwell cell migration analysis was performed using 5 ng/ml TGF-β1 as chemoattractant. Cells on the back of the insert membrane were fixed and stained with 10% May Grunwald-Giemsa. Representative pictures of transwell membrane adherence of HCC cells are shown (x200). For quantification, the average number of migrated

cells per field was assessed. (***) P < 0.001 vs. Control and Ad-βgal; Student's *t* test. All assays were done in triplicate.

Figure 5. Gene transfer of SPARC induces mesenchymal -to- epithelial transition in hepatocellular carcinoma cells.

A) HepG2 cells were infected (at a MOI of 100) with AdsSPARC, AdasSPARC or Adβgal for 3 days and whole-cell lysates were generated. E-cadherin expression was assessed by western blot. A representative of three independent experiments is shown. A goat antibody against actin was used as control of protein loading. **B)** SPARC overexpression induces a decrease of N-cadherin expression in HepG2 cells. HepG2 cells were infected (at a MOI of 100) with AdsSPARC or Ad-βgal for 3 days, fixed and incubated with antibodies to N-cadherin. By using fluorescence-activated flow cytometry analysis, N-cadherin expression is displayed by a shift in mean fluorescent intensity when compared to incubation without addition of any primary antibody. The percentages of N-cadherin positive cells are indicated. **C)** Quantitative analysis from comparisons of results obtained from independent studies similar to that shown in B). **D, E, F, G)** Representative pictures from N-cadherin immunostained HepG2 cells, which were previously transduced with AdsSPARC (D,E) or Ad-βgal (F,G) and plated onto poli-L-lysine/fibronectin coated coverslips. **H, I)** Quantification of the N-cadherin (H) and α-SMA (I) immunostaining intensities in HepG2 cells is represented. The data are expressed as the Mean ± SEM. (*) vs. Control; (τ) vs. Ad-βgal. (*) p<0.05; (ττ) p<0.01; (***,σσσ) p<0.001. Student's *t* test.

Figure 6. Up-regulation of SPARC expression decreases HCC cell viability in response to 5-FU-based chemotherapy and increases apoptosis.

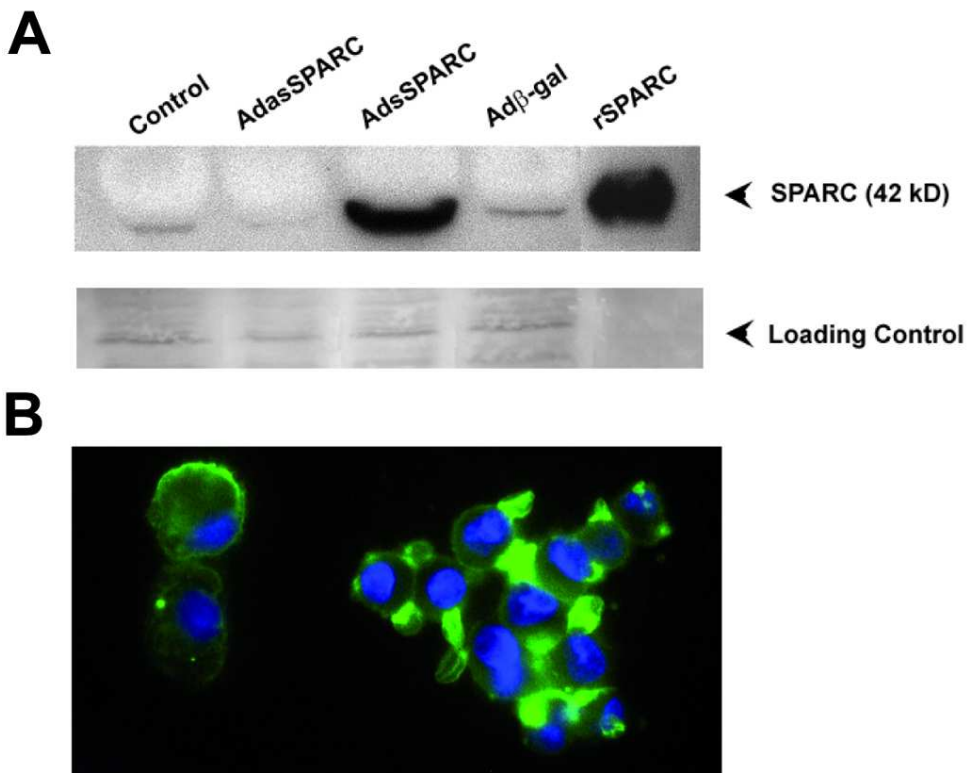
A) HepG2 cells were infected with AdsSPARC, Ad-βgal or left uninfected for 2 days, washed and further incubated with 5-FU for 24 h. Cell viability was assessed by MTT assay (Invitrogen) at 490 nm. The data is expressed as the mean absorbance ± SEM. **B)** HepG2 cells were infected with AdsSPARC, Adβgal or left uninfected for 2 days, washed and further incubated with 5-FU for 24 h and stained with an AO/EB bromide mixture. Percentage of apoptotic cells is shown. The data are expressed as the Mean ± SEM. (*) vs. Control; (σ) vs. Ad-βgal. (*,σ) $p<0.05$; (**,σσ) $p<0.01$; (***,σσσ) $p<0.001$. Student's *t* test.

Figure 7. The *in vivo* growth capacity of SPARC overexpressing HCC cells is strongly inhibited in nude mice.

AdsSPARC significantly increased animal survival. Nude mice were s.c. inoculated with $1.5x10^6$ HepG2 **(A)** or $5x10^6$ Huh7 **(C)** cells transduced with AdsSPARC or AdasSPARC or Ad-βgal or left untransduced and tumor size was measured with caliper twice a week over a period of 42 days. Effects of AdsSPARC treatment on survival of the tumor-bearing nude mice (**B**, HepG2 cells; **D**, Huh7 cells) (Kaplan-Meier survival curve). The data are expressed as the Mean ± SEM. (*) vs. Control; (σ) vs. Ad-βgal. (*,σ) $p<0.05$; (**,σσ) $p<0.01$; (σσσ) $p<0.001$. Mann-Whitney test.

Figure 8. Host macrophage cells are involved in the rejection of SPARC overexpressing HCC cells. Nude mice were s.c. inoculated with $1.5x10^6$ HepG2 cells transduced with AdsSPARC, AdasSPARC, Adβgal or left untransduced. **A)** H&E staining showing a reduced tumoral rim in mice from AdsSPARC group. Arrows: fibroblastic-like cells; dotted arrows: tumor cells. **B)** Representative photographs of immunohistochemical staining of macrophages (F4/80) in HepG2 tumors at 5 days after

inoculation, corresponding to HepG2 (upper, 100x; lower, 400x) and AdsSPARC-HepG2 (upper, 100x; lower, 400x) treated mice. **C)** Quantitative graph showing comparisons of the F4/80 immunostained area in different experimental conditions. The data are expressed as the Mean ± SEM. (*) vs. Control; (σ) vs. Ad-βgal. (*,σ) $p<0.05$; (**,σσ) $p<0.01$; (***,σσσ) $p<0.001$. Mann-Whitney test. **D)** SPARC increases in vivo HCC apoptosis. The effect of SPARC overexpression on apoptosis was assessed by TUNEL assay. Representative photographs of TUNEL assay in HepG2 tumors at day 5 after treatment, corresponding to Adβgal, AdsSPARC, AdasSPARC or untransduced HepG2 cells.



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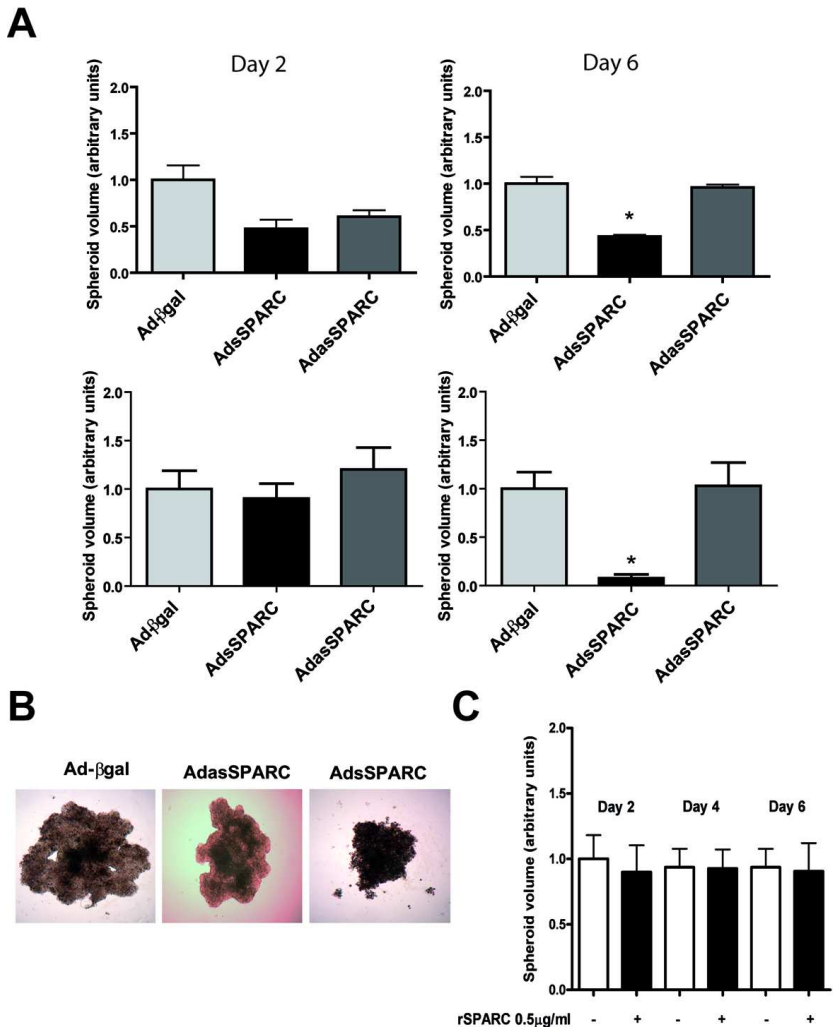

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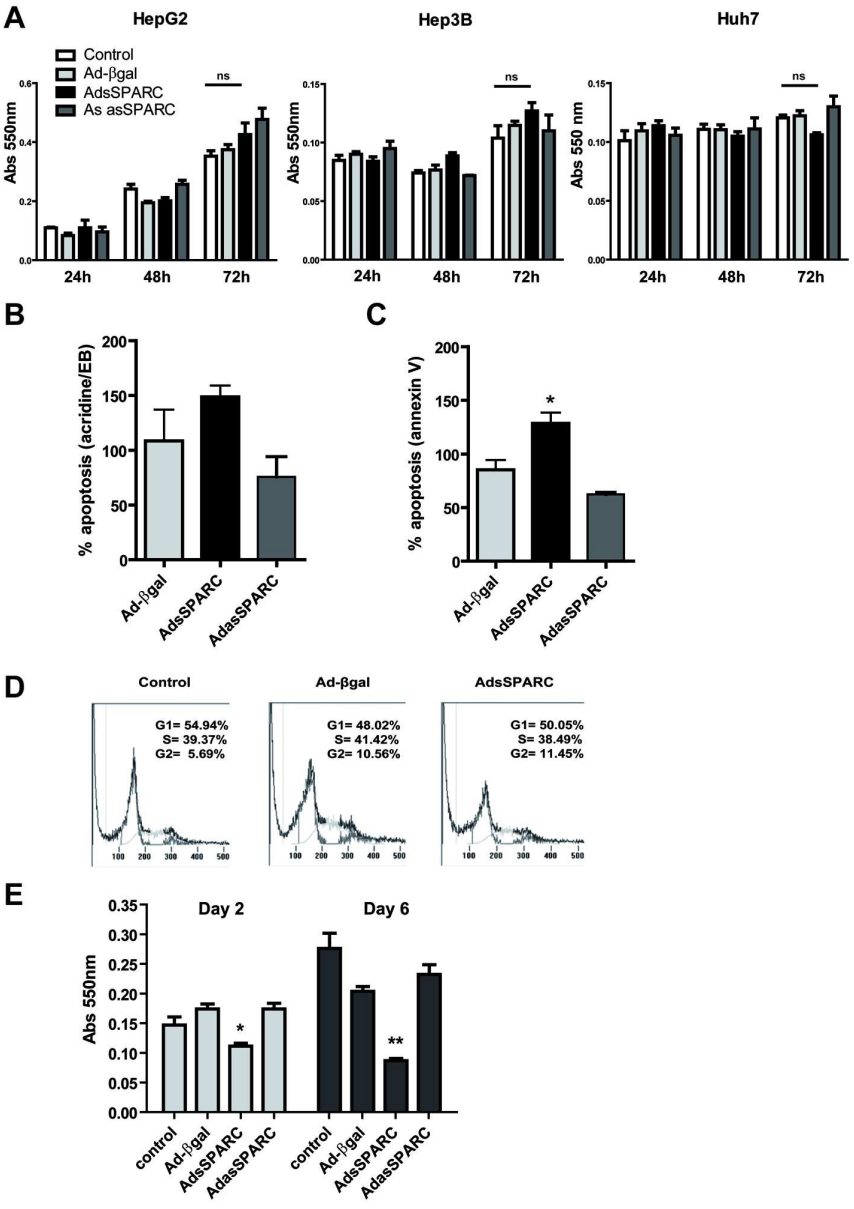
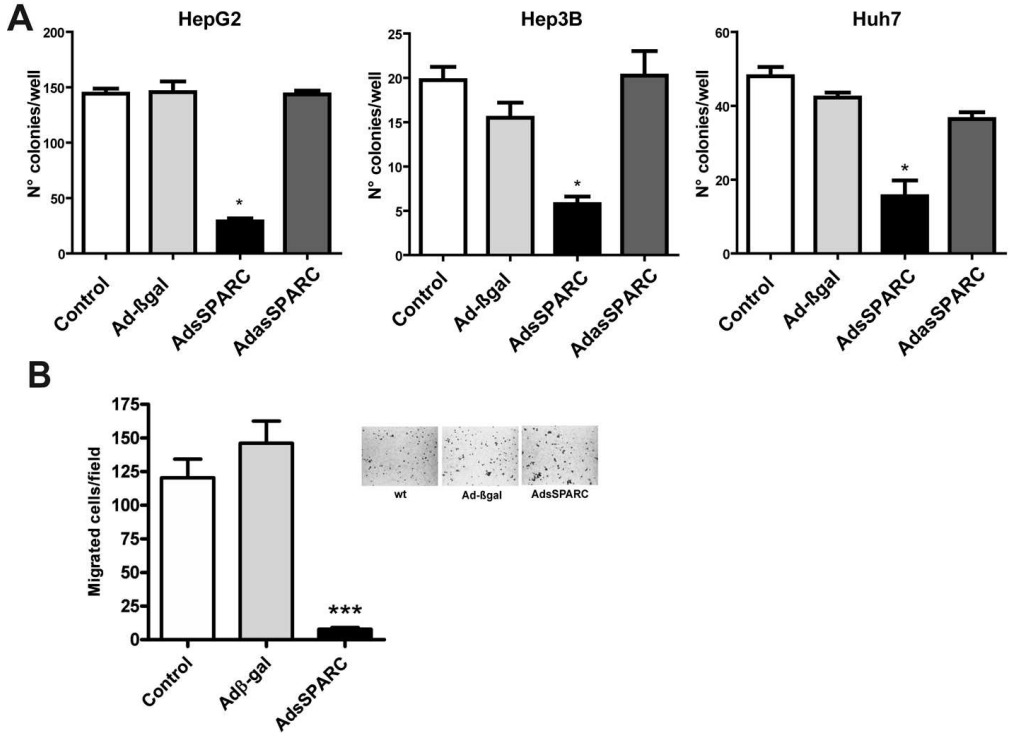
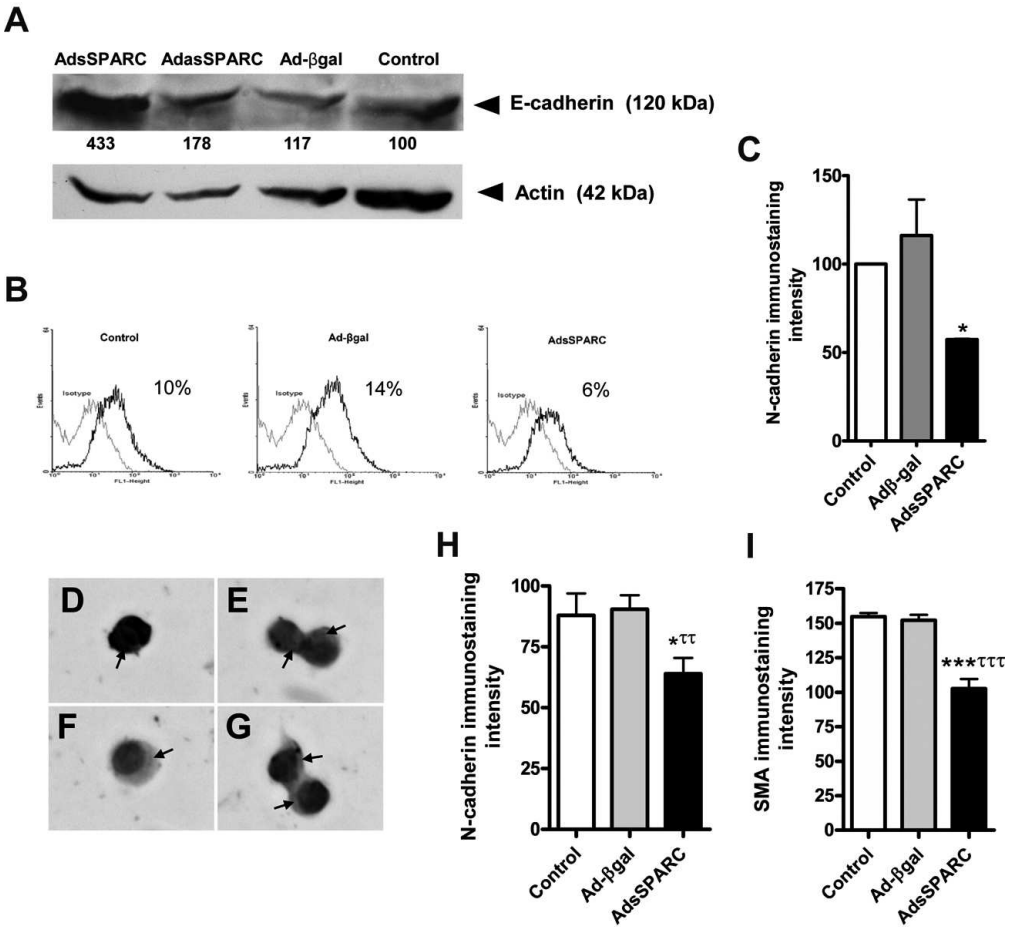


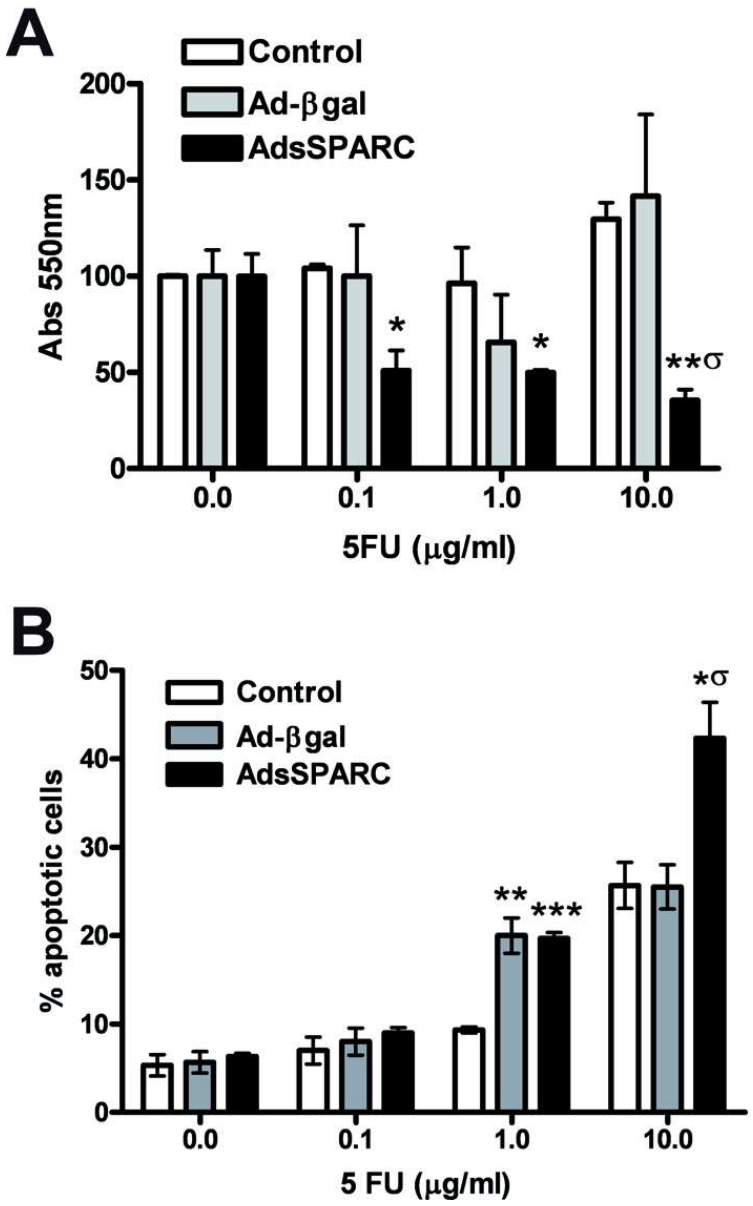
119x169mm (300 x 300 DPI)



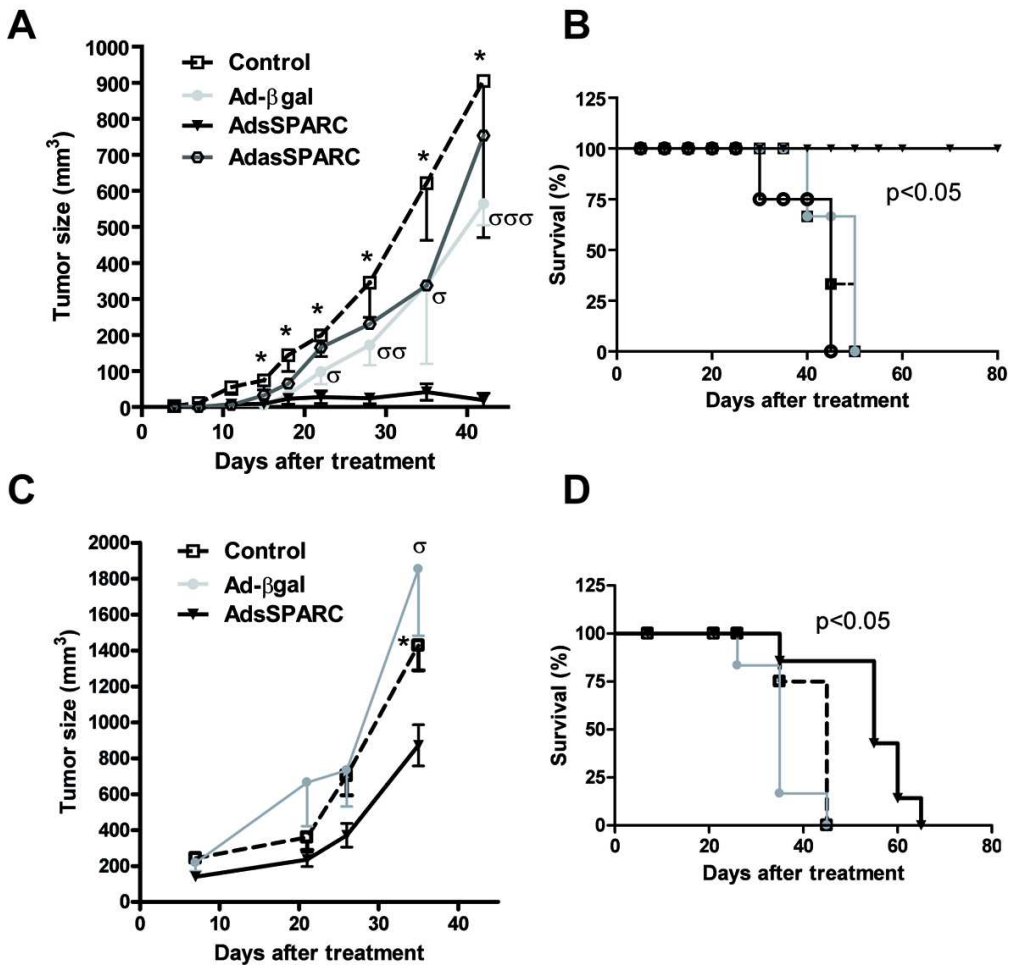
119x88mm (300 x 300 DPI)



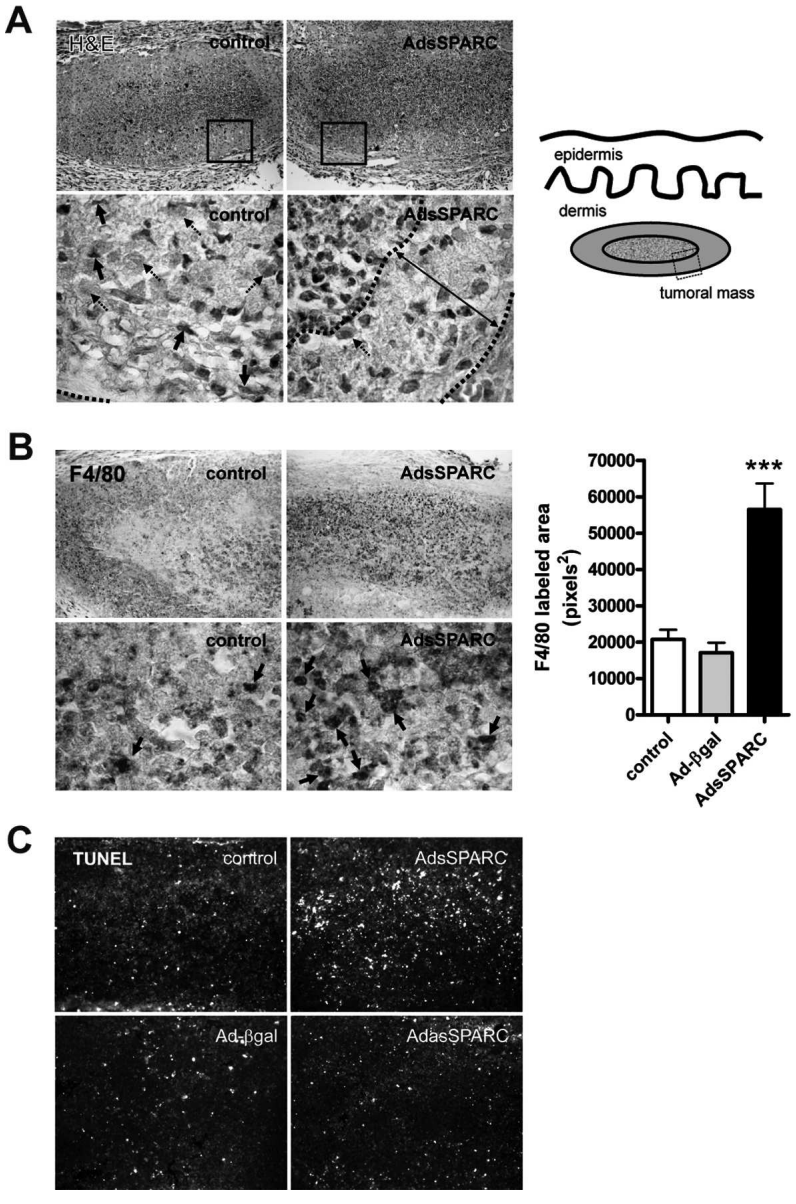
109x102mm (300 x 300 DPI)



60x96mm (300 x 300 DPI)



99x96mm (300 x 300 DPI)



99x151mm (300 x 300 DPI)